Title

Escalation of pyrethroid resistance in the malaria vector *Anopheles funestus* induces a loss of efficacy of PBO-based insecticide-treated nets in Mozambique

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Keywords

Malaria, insecticide resistance, vector control, *An. funestus*, Mozambique, Long Lasting Insecticidal Nets, metabolic resistance, Cytochrome P450.

Summary

An extensive loss of efficacy of all pyrethroid-based Insecticide-treated bednets, including PBO-based bednets is recorded in a highly pyrethroid resistant population of the malaria vector *An. funestus* (Mozambique) exhibiting high expression of cytochrome P450 genes with fixation of CYP6P9a_R allele.

Abstract

Background: Insecticide resistance poses a serious threat to insecticide-based interventions in Africa. There is a fear that resistance escalation could jeopardize malaria control efforts. Monitoring cases of aggravation of resistance intensity and its impact on the efficacy of control tools is crucial to predict consequences of resistance.

Methods: The resistance levels of an *Anopheles funestus* population from Palmeira in southern Mozambique was characterised and its impact on the efficacy of various insecticide-treated nets established.

Results: A dramatic loss of efficacy of all long lasting insecticidal nets (LLINs) including PBO-based nets (Olyset Plus) was observed. This *An. funestus* population consistently (2016, 2017 and 2018) exhibited high degree of pyrethroid resistance. Molecular analyses revealed that this resistance escalation was associated with a massive over-expression of duplicated cytochrome P450 genes, *CYP6P9a/b* and also the fixation of the resistance CYP6P9a_R allele in this population in 2016 (100%) in contrast to 2002 (5%). However, the low recovery of susceptibility after PBO synergist assay suggests that other resistance mechanisms could be involved.

Conclusions: The loss of efficacy of pyrethroid-based LLINs with and without PBO is a concern for the effectiveness of insecticide-based intervention and action should be taken to prevent the spread of such super-resistance.

Background

Malaria burden remains high in Africa (1) despite recent progress achieved mainly through insecticide-based interventions such as long lasting insecticidal nets (LLINs) and Indoor Residual Spraying (IRS) (2, 3). Increasing reports of resistance to major insecticide classes is a worrying concern for the continued effectiveness of insecticide-based control tools. The resistance to pyrethroids is particularly problematic, as it is the main insecticide class approved for LLINs impregnation, as well as the most common insecticide class used in IRS (4). Therefore, devastating consequences are predicted for malaria control if pyrethroid efficacy is lost, as highlighted by the World Health Organization (WHO) (5). However, there is currently an intense debate with opposite results often published about the impact of insecticide resistance on the effectiveness of insecticide-based interventions (6, 7). This contrast is highlighted by the difference observed between a multi-country study showing a lack of impact of pyrethroid resistance on malaria transmission (6) whereas a field trial in Tanzania supported that pyrethroid resistance was reducing the effectiveness of pyrethroid-only LLINs and impacting malaria transmission (7). Among other factors, it is possible that this discrepancy is associated with the variation of the strength of resistance in respective populations studied. Indeed, it is acknowledged that increasing resistance levels (resistance ratio) is more likely to lead to control failure than standard resistance levels (8, 9). This highlights the crucial need to monitor field populations for evidences of resistance escalation and to measure potential impact of resistance escalation on the efficacy of insecticide-based tools including LLINs. However, limited studies have been performed on the escalation of resistance in field populations of malaria vectors in Africa. A study in

Burkina Faso (West Africa) revealed that increase resistance in *Anopheles gambiae* negatively impacted the efficacy of pyrethroid-only nets (10). Similarly, a loss of efficacy of pyrethroid-only nets was observed in a population of *Anopheles funestus* s.s. in southern Mozambique (southern Africa) (11) previously shown to be highly resistant to pyrethroid (11, 12) suggesting that this population could be ideal to monitor the increase in resistance levels and its consequences.

Pyrethroid resistance in *An. funestus* is widespread throughout Mozambique, notably in the south where mosquitoes have been shown to survived 3h exposure to pyrethroids in WHO bioassays (12-16). This resistance is driven by metabolic resistance mediated by over-expression of cytochrome P450s including two duplicated P450s, *CYP6P9a* and *CYP6P9b* (17, 18). The recent detection of a DNA-based marker for *CYP6P9a* resistant allele revealed that the CYP6P9a_R frequency was elevated in southern Mozambique (19). In contrast, to date, no knockdown resistance (*kdr*) mutation in the *voltage-gated sodium channel* (VGSC) gene has been reported in the *An. funestus* s.s. Africa-wide (17, 20, 21). A new generation of LLINs, combining a pyrethroid with the synergist piperonyl butoxide (PBO), has been designed by manufacturers to overcome this growing problem of pyrethroid resistance. PBO inhibits the action of the cytochrome P450s (22, 23), enhancing the effect of pyrethroids on resistant *kdr*-free mosquitoes (24-26). The impact of increased resistance levels in southern Mozambique remains un-elucidated on the efficacy of PBO-based nets. It remains also unknown if the escalation of resistance is associated with over-expression of metabolic resistance genes such as *CYP6P9a/b* and if such increased expression of P450 genes could reduce the inhibition effect of PBO to reduce the efficacy of PBO-based nets.

To fill this gap and facilitate the design of resistance management strategies, we extensively investigated the resistance profile and resistance mechanisms of one, highly resistant population *of An. funestus* s.s. in Southern Mozambique (Palmeira). Our study reveals an extensive loss of efficacy of all pyrethroid-based LLINs tested, including PBO-based LLINs against this population characterised by a high expression of key cytochrome P450 genes coupled with a fixation of the *CYP6P9a* P450 resistance allele.

Methods

Mosquito collection

Indoor female *Anopheles* mosquitoes were collected in the village of Palmeira (25° 15' 19''S; 32° 52' 22''E), Manhiça district, Maputo province (southern Mozambique) near the Incomati river. The majority of inhabitants are farmers (sugar cane, rice) from the Xichangana and Xironga communities. The collection were performed during 4-5 days in three consecutive years (August 2016, April 2017 and January 2018) using electric aspirators. *An. funestus* s.s. is the primary malaria vector in this area (27). *An. funestus* sample collected in 2002 (28) was used for comparative genotyping the *CYP6P9a* resistance allele. Most of the households have LLINs (Olyset and PermaNet 2.0) impregnated only with pyrethroids whereas IRS with dichlorodiphenyltrichloroethane (DDT) is also applied (27).

Collected gravid, blood-fed and half-gravid *Anopheles* females mosquitoes were morphologically identified as belonging to *An. funestus* group or *An. gambiae* complex according to morphological keys (29). Females *An. funestus* sensu lato (s.l.)

were kept in cages until they became fully gravid, and subsequently, forced to lay eggs in separate 1.5 ml microcentrifuge tubes and larvae reared to adults as previously described (30). Seventy *An. funestus* s.l. female mosquitoes collected in April 2017 were bisected into head plus thorax and abdomen and kept individually. Genomic DNA (gDNA) from these mosquitoes were extracted using the Livak method (31) followed by a cocktail polymerase chain reaction (PCR) as previously described (32) for species identification with *An. funestus* group. The internal transcribed spacer 2 (ITS2) was sequenced for samples that failed to amplify.

Plasmodium infection rates

A TaqMan assay was used to screen for *Plasmodium falciparum* (P*f*) and *P. ovale, P. vivax and P. malariae* (P*ovm*) in 57 heads plus thoraxes gDNA (sporozoite) from 2017 F_0 *An. funestus* s.s. females as previously described (33, 34). Subsequently, a nested PCR (35) was also performed to validate all the *Plasmodium* positive samples.

Insecticide-treated bed nets efficacy assays

Following the WHO guidelines for cone bioassays (36), the effectiveness of the following LLINs was estimated for: Olyset® Net (permethrin 2%) and Olyset® Plus net roof (permethrin 2% plus PBO 1% in the roof) ; PermaNet® 2.0 (deltamethrin 0.18%) and PermaNet® 3.0 side (deltamethrin 0.28%). An untreated mosquito net was used as a control. Five replicates of ten F_1 2–5 days old females were placed in plastic cones enclosed with the mosquito net during 3 min exposure. Mosquitoes were then placed in small holding paper cups with cotton soaked in 10 % sugar solution. Mortality was determined 24 h later in 2016 and 2018, and every 24

hours, until five days in 2017. The efficiency of the LLINs was confirmed using the *An. gambiae* susceptible laboratory strain, Kisumu (2016 and 2017) and the *An. funestus* susceptible FANG strain (2018).

Insecticide susceptibility assays

The insecticide resistance profile of *An. funestus* s.s. were assessed using the WHO tube bioassays (37). *An. funestus* s.s. mosquitoes collected in 2016 were tested to the pyrethroids type I permethrin (0.75%) and type II deltamethrin (0.05%), the organochlorine DDT (4%), and the carbamate bendiocarb (0.1%). Mosquitoes collected in 2017 were additionally tested with the pyrethroid derivative, etofenprox (0.05 %) and the organophosphate malathion (5%). Assays were performed at 25 ± 1° C and 70-80% relative humidity. At least three replicates of 20-25 F_1 female and male mosquitoes 2-5 day-old were exposed separately to insecticide-impregnated papers for 1h and afterwards transferred to a holding tube provided with cotton soaked in 10 % sugar solution. Mortality was determined 24h later. Control tubes using carrier oil-impregnated papers were performed for each bioassay. Synergist assays with piperonyl butoxide (PBO; inhibitor of cytochrome P450s) was performed as previously described (38).

Additionally, due to the extremely high resistance to permethrin, an insecticide commonly used in LLINs, the intensity of resistance was assessed by exposing three replicates of 20-25 F_1 female mosquitoes for 90, 120 and 180 minutes to permethrin in WHO tube bioassays as described above.

Transcription profile of resistance genes in *An. funestus* s.s.

Total RNA was extracted from 3 batches of 10 adult 2-5 days old F_1 females *An. funestus* s.s. non-exposed to insecticides and similarly from the susceptible laboratory strain FANG, as previously described (17). The transcription patterns of the duplicated cytochrome P450 genes *CYP6P9a* and *CYP6P9b*, major pyrethroid resistance genes in this region (17, 39), plus the DDT/permethrin resistant gene-related *glutathione-s-transferase epsilon 2* (*GSTe2*) (40), were assessed by a quantitative reverse transcription PCR (qRT-PCR), as previously described (17, 41). The relative expression was calculated individually according to the 2-ΔΔCT method (42) and compared to that previously published in southern (Malawi), West (Ghana and Benin), East (Uganda) and Central (Cameroon) Africa (43).

Genotyping of the CYP6P9a_R pyrethroid resistance allele

A PCR-RFLP assay recently designed (19) was used to genotype the CYP6P9a_R allele in Palmeira in 2016 and 2017 but also in 2002 to assess potential link between the aggravation of resistance and this allele.

Genotyping of other resistance markers in *An. funestus* s.s.

The presence of other *An. funestus* s.s. resistance markers was assessed including N485I-Ace-1 (bendiocarb), A296S-RDL (dieldrin) and L119F-GSTe2 (DDT/permethrin) using TaqMan assays, as previously described (44, 45) (N485I-Ace-1 and A296S-RDL) and an allele-specific PCR (AS-PCR) (L119F-GSTe2) (46).

Results

Species identification

750, 1100 and 425 F_0 female mosquitoes were sampled respectively in 2016, 2017 and 2018 with 40-50% blood fed, half or fully gravid. 243, 360 and 185 females laid eggs respectively in 2016, 2017 and 2018 with 120, 250 and 92 hatching. From 96 (2016), 70 (2017) and 50 (2018) F_0 females *An. funestus* s.l. molecularly assessed by PCR, 90, 57 and 50 females were identified as *An. funestus* s.s. respectively, while 6 (2016) and 13 (2017) failed to amplify. Subsequently, through sequencing of the ITS2, the 13 samples (2017) were also identified as *An. funestus* s.s.

Plasmodium sporozoite infection rate

The *Plasmodium* sporozoite-infection rate in *An. funestus* s.s. (2017) was 5.3% (3/57). Two *An. funestus* s.s. females were infected *with P. falciparum* sporozoites (3.5%, 2/57), and one infected with ovm+ (1.8%, 1/57). A nested PCR confirmed all the positive infected mosquitoes and determined that the ovm+ positive sample was infected with *P. malariae*.

Insecticide-treated bed nets efficiency

No mortality was recorded following 3-minute exposure for all LLINs tested including Olyset Plus PBO-based net against F_1 females collected in 2016 suggesting an extensive loss of efficacy of these nets. However, the same nets induced a total mortality against the control Kisumu susceptible *An. gambiae* mosquitoes (Figure 1A). To confirm these results, a new batch of LLINs was tested against another sample of *An. funestus* s.s. collected in 2017 revealing similar loss of efficacy with mortalities less than 6.2% after 24 h. Due to this exceptional loss of efficiency the mortality was also monitored at 48, 72, 96 and 120 h after exposure in 2017 (Figure 1B). After 120 h, similar low mortalities (between 5.7 and 7.5%) were recorded for

both LLINs impregnated with deltamethrin (PermaNet® 2.0 and 3.0 (side)) and control, while the two LLINs impregnated with permethrin (Olyset® Net and Olyset® Plus) presented a slightly higher mortality (12 and 13.6%, respectively). A similar loss of efficacy was observed in 2018 (Figure 1C) in contrast to the high mortality in FANG, the *An. funestus* laboratory susceptible strain. These results indicate a surprisingly extensive loss of action of the PBO in the PBO-based LLINs (Olyset Plus) against this *An. funestus* population. Mortality rate with the control net was 0% in 2016 and 2018 and 2% in 2018.

Insecticide susceptibility assays

F_1 females from both collections exhibited an extremely high resistance to permethrin (pyrethroid type I; used in Olyset® nets) and deltamethrin (pyrethroid type II; used in PermaNet® nets), supporting the observed loss of efficiency of LLINs (Figure 2A and 2B). F_1 females collected in 2016 showed no mortality after 24h exposure to both pyrethroids tested (permethrin and deltamethrin) (Figure 2A), while F_1 females collected in 2017 showed 13.9 ± 2.4% and 12.3 ± 4.3% mortality to permethrin and deltamethrin, respectively (Figure 2B). F_1 females collected in 2017 also presented high resistance to the pyrethroid derivative, etofenprox (5.9 ± 0.2% mortality and to the carbamate bendiocarb, with mortalities of 42.3 ± 6.3% and 29.8 ± 11.4% in 2016 and 2017, respectively (Figure 2B). However, it consistently showed a full susceptibility to the organochlorine, DDT and the organophosphate, malathion with 100% mortality rates. Male mosquitoes (2017) exhibited similar resistance profile (Figure 2B).

Synergist assays performed in 2017 with PBO revealed only a moderate recovery of susceptibility after exposure to permethrin and deltamethrin [permethrin:

no PBO pre-exposure 13.9 ± 2.4% mortality *versus* PBO pre-exposure 29.7 ± 5.8%, *P=0.065*; deltamethrin: no PBO pre-exposure 12.3 ± 4.3% *vs.* PBO pre-exposure 22.3 ± 15.9%, *P=0.24*] (Figure 2C). Tests with bendiocarb also revealed a lack of impact of PBO pre-exposure with no difference in mortality [27.9 ± 4% mortality (P = 0.09) with PBO exposure *vs.* 29.8 ± 11% without exposure. No mortality was observed in control mosquitoes exposed to the synergist PBO only.

Due to the high resistance observed to pyrethroids, WHO tube bioassays with F_1 females collected in 2017 and exposure times of 90, 120 and 180 min to permethrin were also performed (Figure 2D). A constant increase in mortality was observed proportional to the time of the permethrin exposure with LT50 estimated at 1h 45min (95% CI 1h 37min–1h 51min).

Transcription profile of resistance genes in *An. funestus* s.s.

Transcription analysis of the duplicated P450 genes *CYP6P9a* and *CYP6P9b*, known to confer pyrethroid resistance in *An. funestus* (17, 39) reveals a high up-regulation of *CYP6P9a* (fold change, FC=122.4 ± 34) and *CYP6P9b* (FC=106.2 ± 32) from Southern Mozambique compared to the susceptible FANG strain (P<0.001). This over-expression is higher than in Malawi (FC=85.5 ± 20.7 and 79.9±11.6 respectively for *CYP6P9a* and *CYP6P9b*) (Figure 3A) although not statistically significant (P>0.05). A greater contrast is observed with other African regions where the over-expression of these two genes is significantly much lower (P<0.001) (Figure 3A). In contrast *GSTe2*, conferring DDT/permethrin resistance in West/Central Africa (40), is not significantly upregulated in Mozambique (FC=1.1 ± 0.4; P=0.84).

Frequency of the CYP6P9a_R allele

Genotyping of 50 (2016) and 57 (2017) F_0 females using the novel PCR-RFLP assay (19) revealed that this resistance allele for P450-mediated metabolic resistance is fixed in Palmeira with 100% of the RR genotype detected in both samples. This contrasts with 2002 (35 females) where the CYP6P9a_R allele was only recorded at 5% (Fig. 3B) (χ^2=1900; P=0.0) suggesting that, beside other factors including increased over-expression of P450s, the escalation of resistance could have been associated with the fixation of the CYP6P9a-R allele in field population.

Frequency of other resistance alleles

The frequency of the 119F-GSTe2 resistant allele was very low (7.4%) (1RR, 6RS and 47SS) (Figure 3C). This is the first detection of this resistance marker in southern Africa as it was completely absent from samples collected in 2010 (40).

The frequency of the A296S-RDL mutation conferring dieldrin resistance (47) was very low (0.9%) (0RR, 1RS and 56SS) (Figure 3C). However, this is its first report in southern Africa.

The N485I mutation in the in *acetylcholinesterase 1* (ace-1) gene associated with bendiocarb resistance (45) was detected at a frequency of 23.9% (5 RR-485I, 18 RS-N485I and 34 SS-N485)(Figure 3C).

Discussion

Assessing the dynamic of resistance to insecticides in major malaria vectors and its impact on the effectiveness of control tools is a key prerequisite for the implementation of suitable strategies to manage the growing challenge from insecticide resistance in malaria control.

This study revealed a complete loss in efficacy of the two most common commercial LLINs used across Africa, Olyset® Net and PermaNet® 2.0 against *An. funestus* s.s. from Southern Mozambique confirming the extensive loss reported for these nets in 2015 in the same region (11). A similar extensive lost was also reported Malawi (<5% mortality) (44) and in DR Congo (<35% mortality)(38). This loss in efficacy in Palmeira is further supported by the WHO insecticide susceptibility assays results, showing a high resistance of this population to permethrin and deltamethrin, the pyrethroid compounds used to impregnate these nets. This high pyrethroid resistance is in line with previous reports in this region (11, 12).

More alarmingly, this study also reported the extensive loss in efficacy of the new generation of PBO-based nets, prior to the implementation of PBO-based bednets in the area. This is the first report of such loss of efficacy of this new generation nets against *An. funestus* as higher mortality rates (>80%) have so far been observed when testing PBO-based nets (Olyset Plus) against other pyrethroid resistant populations including in Malawi (44) and DR Congo (38). The extensive ability of mosquitoes to survive exposure to PBO-based nets in this population also differs significantly from results in *An. gambiae* for which highly resistant populations have shown mortality of around 40% such as in Burkina Faso (10) and DR Congo (38). However, the PermaNet 3.0 Top (containing PBO) was not analysed. Nevertheless, the low mortality with PermaNet 3.0 (side) here contrary to the higher mortality rate (88%) observed in DRC suggests also a loss of efficacy of PermaNet 3.0. In this study, we did not analyse the chemical content of the nets (using HPLC) to confirm that the quantity of PBO or pyrethroids on the nets tested are those stipulated by the manufacturers. Such work will need to be done in the future as one cannot rule out

some loss of the active ingredients in the nets. However, when checking these same nets against the susceptible lab strains (Kisumu and FANG), we observed a total mortality supporting the loss of efficacy against the field resistant *An. funestus* populations.

The low mortality against PBO-based nets of the *An. funestus* population of southern Mozambique is further remarkable as this population does not possess the knockdown resistance (*kdr*) (21, 28) contrary to the *An. gambiae* VK7 population from Burkina Faso. The lack of *kdr* in this Mozambican population is further supported by a total susceptibility of this population to DDT, a chemical which also targets the sodium channel gene. Previous studies and the qRT-PCR performed here indicate a predominant role of metabolic resistance in this *An. funestus* population primarily driven by the duplicated P450s *CYP6P9a* and *CYP6P9b* (17). It is possible that the reduced efficacy of all bed nets including PBO-based is partly due to a dramatic over-expression of these P450s that could allow mosquitoes to withstand exposure to pyrethroids even with the amount of PBO present in the nets. This is supported by the highest level of expression observed in this study in Mozambique for *CYP6P9a* and *CYP6P9b* compared even to another southern African population in Malawi but even more when compared to other regions in East, Central and West Africa. The role of *CYP6P9a/b* is further supported by the observation that the resistant allele of *CYP6P9a*, which has been demonstrated to have a higher catalytic efficiency in metabolising pyrethroids than other alleles (39), has now been driven to fixation in this population whereas it was only present at 5% back in 2002. Such selection was recently shown to be driven by scale-up of insecticide-based interventions in the region including pyrethroid-based IRS and LLINs (28). Therefore,

it is possible that the concentration of PBO in the LLINs is no more enough to inhibit the action of the highly over-expressed and metabolically efficient *CYP6P9a*/*CYP6P9b* alleles in resistant mosquitoes from Palmeira.

However, the low recovery of mortality observed here after exposure to PBO during synergist assay, besides massive over-expression of P450s such as *CYP6P9a/b*, could also be due to other mechanisms that are present in this *An. funestus* population. A potential role of the reduced penetration due to thickening of the mosquito's cuticle was previously suggested in the laboratory strain FUMOZ-R originated from southern Mozambique (48). Such mechanism could also be acting in field population of southern Mozambique contributing to the high resistance to pyrethroids and the loss of efficacy observed in combination with high over-expression of highly efficient P450 enzymes. However, future studies are needed to elucidate the molecular basis of this resistance escalation that is inducing such loss of efficacy of all LLINs including PBO-based nets.

The results of this study support the IRS interventions in Southern Mozambique particularly based on organophosphates. Furthermore, the full susceptibility to DDT supports the lack of over-expression of the glutathione-S transferase *GSTe2* gene in this population contrary to those in West and central Africa (19, 40). However, this study detected for the first time in Southern Africa the resistant allele, 119F-GSTe2, frequently present in West and Central Africa (40). The increase frequency of this allele in southern Africa in combination to high over-expression of cytochrome P450 genes could lead to super resistance to pyrethroids and also DDT.

Conclusions

The loss in efficiency of new generation PBO-based LLINs against *An. funestus* s.s. reported here represents a serious challenge for its future implementation in southern Mozambique. The spread of the molecular mechanisms that confer this "resistance" to the synergistic effects of PBO to other vector populations is an even a more worrying concern. This study highlights the urgent need to investigate the causes of the loss in efficacy of PBO-based nets and to monitor the spread of such operationally significant resistance in other mosquito populations and assess its impact on malaria transmission. Furthermore, efficacy of PBO-based nets should be assessed prior to the rolling out of these nets in Mozambique.

Funding

This work was supported by a Wellcome Trust Senior Research Fellowship in Biomedical Sciences to Charles S Wondji (101893/Z/13/Z).

Acknowledgments

Silvie Huijben was supported by the Society in Science - Branco Weiss Fellowship. KPP was supported by the Bill and Melinda Gates Foundation and Obra Social "la Caixa" Partnership for the Elimination of Malaria in Southern Mozambique (OPP1115265). ISGlobal is a member of the CERCA Programme, Generalitat de Catalunya.

Conflict of interest

All the authors declare that there is no conflict of interest concerning the research conducted and the publication of the results obtained.

Author's contribution

C.S.W designed the study; J.M.R, S.H. and K.P.P. supervised the fieldwork; N.C. and M.M. contributed to field work; J.M.R, W.T. and M.T. performed the resistance testing in the insectary, M.J.W., M.T. J.M.R, H.I and C.S.W performed the molecular experiments; J.M.R and C.S.W. analyzed the data; J.M.R and C.S.W wrote the manuscript with contribution from all the authors. All authors read and approved the final manuscript.

Previous presentations

The content of the manuscript is original and the information has not previously been presented in conferences, meetings or elsewhere.

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Figure legends

Figure 1: Bio-efficacy of different commercial LLINs against *An. funestus* s.s. in Palmeira. A) is for 2016, B) for 2017 and C) for 2018. Error bars represent standard error of the mean.

Figure 2: Susceptibility profile of *An. funestus* population from Palmeira, southern Mozambique: A) susceptibility profile in 2016 for females only; B) Susceptibility profile in 2017 for both males and females. C) Synergist assay with piperonyl butoxide (PBO) (n=4); D) Time-point mortality rates for permethrin with estimation of LT50 at 1h 45min. Error bars represent standard error of the mean.

Figure 3: Exploration of the molecular basis of the escalation of pyrethroid resistance in *An. funestus*: A) Comparative gene expression of the P450 genes *CYP6P9a* and *CYP6P9b* in Mozambique comparatively to other African regions; Error bars represent standard error of the mean. B) Allele frequency of the *CYP6P9a* pyrethroid resistance marker in southern Mozambique populations from 2002, 2016 and 2017 showing a fixation of the CYP6P9a_R allele. C) Distribution of the genotypes of resistance markers in Palmeira including CYP6P9a_R, L119F-GSTe2, N485I-Ace-1 and A296S-RDL.

Figure 1

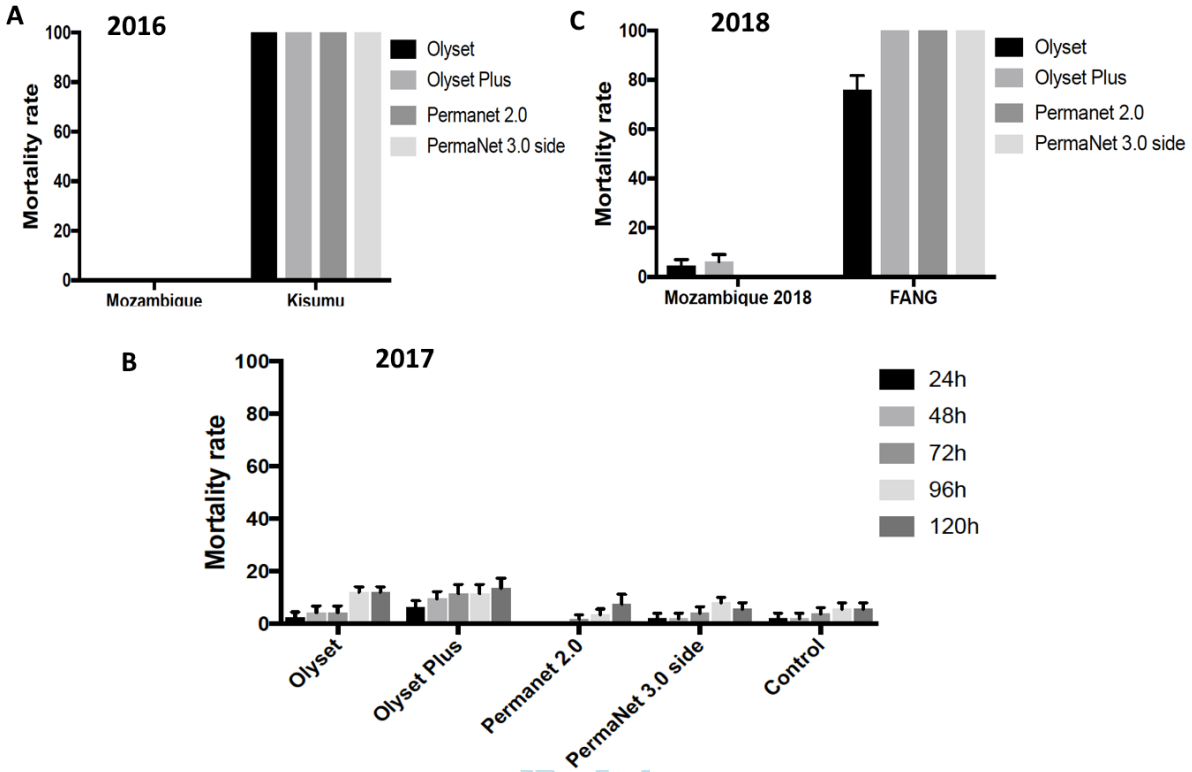

Figure 2

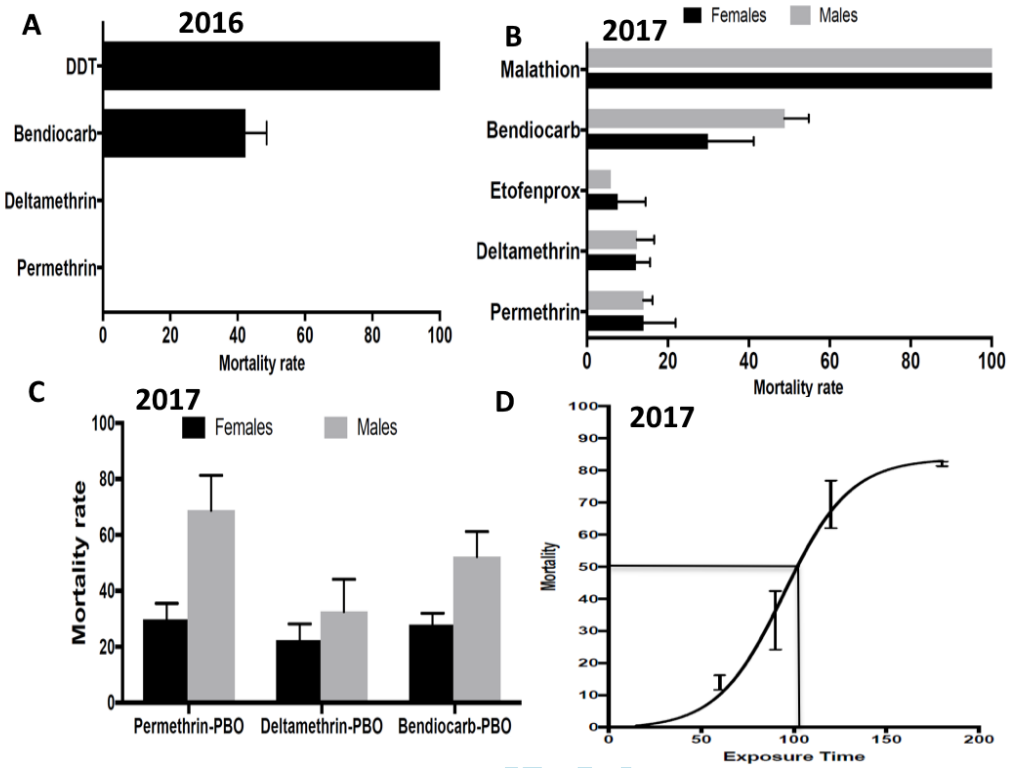

Figure 3